An Investigation of *Coprinus atramentarius* for the Presence of Disulfiram*

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Coprinus atramentarius Fries was grown in surface culture on selected media. The quantity of mycelium formed was small; no carpophores were produced. Addition of a low concentration of disulfiram to cultures of the fungus had no apparent effect on the development of the organism, and the added disulfiram disappeared from the cultures within sixty days. Concentrated extracts of the media and mycelia of the cultures of the fungus, as well as extracts of naturally-occurring carpophores, were investigated for the presence of disulfiram by means of a paper partition chromatographic method. It is concluded that the disulfiram-like physiological activity which was exhibited by the naturally-occurring carpophores of *C. atramentarius* is due to some agent other than disulfiram.

IT HAS LONG been known that ingestion of the mushroom *Coprinus atramentarius* Fries and the subsequent ingestion of alcohol gives rise to physiological symptoms very similar to those of the alcohol-disulfiram syndrome. Reports of *Coprinus*-alcohol poisoning are common in the mycological literature (1–5) and antedate considerably the discovery of the alcohol-disulfiram syndrome. The remarkable similarity between the two types of poisoning has given rise to considerable speculation that disulfiram might exist in *C. atramentarius* (4).

In 1956 two Czechoslovakian investigators, J. Simandl and J. Franc (6), reported the isolation of disulfiram from *C. atramentarius*. They extracted autolyzed carpophores of the fungus with carbon tetrachloride in the cold, evaporated the carbon tetrachloride extract, and extracted the resulting residue with anhydrous methanol. Evaporation and recrystallization yielded a crystalline substance with a melting point of 70.5°, the melting point of disulfiram, and this substance had the properties of disulfiram when subjected to paper partition chromatographic procedures. They did not attempt a quantitative determination.

Since the physiological action of the combination of alcohol and *C. atramentarius* suggested the possible presence of disulfiram in that fungus, and since the presence of disulfiram in the fungus had been reported by Simandl and Franc, it seemed of interest to determine if quantities of the organism could be produced successfully in culture and if the fungus would produce disulfiram when so grown. The culture of *C. atramentarius* used to initiate this investigation was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Although numerous hymenomycetes, including a number of *Coprinus* species, have been successfully grown in the laboratory, a recent review (7) revealed that the culture of *C. atramentarius* had been studied by only one investigator, C. H. Chow (8).

A number of media have been employed for culture of *Coprinus* species (9–13). Fresh horse dung or extract of fresh horse dung, alone or in combination with other nutrients, was the substrate most commonly used. Chow used fresh horse dung as well as other media in his cultures of *C. atramentarius*. He maintained only a few cultures of this fungus, as the spores germinated with difficulty and in low percentage, and he reported that the organism did not produce normal carpophores in artificial culture.

During the course of the present investigation it became obvious that *C. atramentarius* was not producing disulfiram when grown on the media employed, and it was decided to investigate the effect on the development of the fungus of adding disulfiram to the culture medium.

Locally-occurring carpophores of *C. atramentarius* were also investigated for presence of disulfiram. In the spring of 1957 a case of typical *Coprinus*-alcohol poisoning had occurred in Seattle, Washington (14). The victim, R. Levin, a University of Washington student, obtained the mushrooms which he ate from the exact site as that from which the mushrooms employed in this investigation were obtained. The growth habit of *C. atramentarius* is such that all these carpophores may be presumed to have arisen from the same mycelial source. Identification of the naturally-occurring carpophores of *C. atramentarius* employed in this investigation was made by Professor D. E. Stuntz, Department of Botany, University of Washington.

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EXPERIMENTAL

Culture of the Organism.—Seven media were originally employed for culture of *C. atramentarius* on a small scale. These were either media used previously for culture of macrofungi by other investigators or modifications of such media made by the authors in an attempt to improve the development of *C. atramentarius*. The media employed were the following:

Medium No. 1

Mannitol	2.0%
Casein hydrolysate	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

This medium was used for the culture of various strains of *Claviceps purpurea* (15), and it has recently been employed for the culture of *Panaeolus campanulatus* by V. E. Tyler, Jr. (16).

Medium No. 2

Glucose	2.0%
Casein hydrolysate	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

Medium No. 3

Glucose	2.0%
Casein hydrolysate	1.0%
Yeast extract	1.0%
$FeSO_4 \cdot 7H_2O$	0.01%
Basic nutrient solution (15)	10.0%
Distilled water	*q. s.*

Medium No. 4

This consisted of Medium No. 3 to which thiamine hydrochloride (200 mcg./L.) was added.

Medium No. 5 (Modified McCrea's medium)

Maltose	1.25%
Peptone	0.125%
Yeast extract	1.25%
KH_2PO_4	0.25%
$MgSO_4 \cdot 7H_2O$	0.125%
Distilled water	*q. s.*

This medium has been employed for the culture of *Claviceps purpurea* (17) and gave satisfactory development of *C. atramentarius* in slant tube cultures.

Medium No. 6 (Modess medium)

Glucose	0.5%
Malt extract	0.5%
KH_2PO_4	0.05%
$MgSO_4 \cdot 7H_2O$	0.05%
NH_4Cl	0.05%
$FeCl_3$	0.005%
Distilled water	*q. s.*

This medium has been used by the Centraalbureau voor Schimmelcultures for the culture of *Boletus luridus* and *Amanita muscaria* (18).

Medium No. 7

Maltose	0.5%
K_2HPO_4	0.025%
$MgSO_4 \cdot 7H_2O$	0.05%
$Ca(NO_3)_2 \cdot H_2O$	0.05%
Horse dung extract	33.33%
Distilled water	*q. s.*

The horse dung extract was prepared by boiling 1 Kg. of fresh horse dung with 1 L. of water for five minutes, straining, and filtering the product, and making to a volume of 1 L. with water. This medium was used by Bille-Hansen (12) for the culture of a number of *Coprinus* species.

For the small scale cultures, 125-ml. portions of the various media were introduced into Roux-type culture flasks. These flasks of media were then sterilized by autoclaving, cooled, and inoculated with the fungus. The inoculated flasks were placed in a constant temperature cabinet maintained at 25° ± 1°. The cabinet had glass doors, and the cultures were exposed to the indirect artificial illumination of the room in which the cabinet was located. The most rapid growth of the organism occurred in medium No. 7, the horse dung extract medium, and it was selected for routine use.

Subsequent cultures were maintained in both the Roux-type culture flasks and in diphtheria toxin culture bottles, the latter containing 1,200 ml. of medium. These cultures were also kept in the constant temperature cabinet. All cultures were harvested when visible growth of mycelium had ceased, a period of sixty to ninety days.

The medium of each culture was separated from the mycelium by straining through a coarse mesh sieve and was then processed to determine the presence or absence of disulfiram. The mycelium so collected was placed in porcelain evaporating dishes and dried in a forced air oven at 45° for three days. The mycelia from cultures grown on the same medium and inoculated on the same day were combined and weighed. The dried mycelium was then investigated for presence of disulfiram.

The development of the fungus was very sparse in all the media. Table I presents a summary of data on the growth of the fungus in the seven media, and the ratios of weight of dried mycelium to volume of medium.

TABLE I.—MYCELIAL YIELDS OF *Coprinus atramentarius* OBTAINED IN DIFFERENT MEDIA

Medium No.	No. of Cultures	Volume of Medium Processed, L.	Total Weight Dried Mycelium, Gm.	Dry Weight of Mycelium, mg./ml. of Medium
1	12[a]	1.500	0.99	0.66
2	8[a]	1.000	0.75	0.75
3	8[a]	1.000	0.82	0.82
4	8[a]	1.000	0.64	0.64
5	16[a]	2.000	1.47	0.74
6	8[a]	1.000	0.65	0.65
7	19[a]	2.325	2.40	1.03
7	13[b]	15.600	12.05	0.77

[a] Roux flask cultures. [b] Diphtheria toxin flask cultures.

To determine whether disulfiram had any effect upon the growth of *C. atramentarius*, twenty 250-ml. Erlenmeyer flasks containing 75 ml. of medium No. 7 to which 0.167 mg. % of disulfiram had been added were inoculated with the fungus. The concentration of disulfiram which could be added was limited by its extremely low water solubility. Another series of 10 flasks of medium No. 7 to which no disulfiram was added was inoculated with the fungus.

An additional 10 flasks of medium No. 7 containing the disulfiram were prepared but not inoculated. The two series of flasks which had been inoculated with the fungus were harvested at the end of sixty days. The total weight of dried mycelium from the 20 cultures with added disulfiram was 2.55 Gm. (1.70 mg. mycelium per ml. of medium). The total weight of the dried mycelium from the 10 cultures with no added disulfiram was 1.30 Gm. (1.73 mg. mycelium per ml. of medium). The added disulfiram apparently had no effect on the development of the fungus.

Five of the 10 flasks of uninoculated medium containing added disulfiram were tested for the presence of that compound shortly after they were prepared. The other five flasks of medium in that series were similarly tested after sixty days. Disulfiram was detected in both cases. Apparently prolonged solution in the medium did not cause significant decomposition of the disulfiram. Neither the medium nor mycelium of the 20 cultures to which disulfiram had been added contained detectable quantities of disulfiram at the end of the sixty-day growth period. The fungus had apparently decomposed the added disulfiram.

When medium No. 7 which had been solidified by the addition of 2% agar was inoculated with the fungus, a sparse feathery growth of mycelium occurred. Small darkened areas of mycelial compaction occurred which exuded a small amount of liquid similar in appearance to the honeydew of ergot. No carpophores were produced.

Detection of Disulfiram.—Disulfiram was separated from semipurified extracts by paper partition chromatography and rendered visible on the sheets by treatment with a suitable reagent. An attempt to utilize the general procedure of Divatia, Hine, and Burbridge (19) for the spectrophotometric determination of disulfiram in blood proved unsuccessful when applied to disulfiram in medium No. 7.

The chromatographic procedure chosen was a modification of that employed by Simandl and Franc (6). Sheets of Whatman No. 1 filter paper, 22 cm. × 56 cm., were immersed for a few seconds in a mixture of kerosene and benzene (1 to 9). The sheets were allowed to air-dry for five minutes and were then reimmersed in the kerosene-benzene mixture. After another five minute drying period, spots of the solutions to be investigated were applied along a line 12 cm. from one end of the kerosene-impregnated sheets of paper. Total amounts of solution varying from 0.25 to 0.50 ml. were applied to the spots in successive 10- to 20-μL. portions. Each portion was allowed to dry thoroughly before the next was applied. The spotted sheets were then placed in a glass chromatographic chamber, and the chromatograms were formed in the descending direction with a solvent system composed of 65% ethanol in water. The atmosphere of the chamber was allowed to equilibrate with the solvent for twenty-four hours prior to the introduction of the chromatograms. After drying, the chromatograms were sprayed with a saturated aqueous solution of cuprous chloride. As this reagent dried, greenish-yellow spots appeared where disulfiram was present. After spraying, these spots appeared dark purple under an ultraviolet lamp, but they did not fluoresce.

In this system, the R_f values obtained for disulfiram ranged from 0.70 to 0.74. Tailing was not objectionable, and the area of the disulfiram spot at the end of its travel was no more than two to three times the area of the original spot. A 100-mcg. quantity of disulfiram applied to the paper could be detected readily after formation of the chromatogram

Each of the concentrated extracts obtained from medium, mycelium, or carpophores was applied to the sheets in amounts of 0.25 and 0.50 ml. Each extract was also applied in a mixed spot with a 150-mcg. quantity of known disulfiram. Control spots of disulfiram[1] were also applied to each sheet.

Extraction Procedures.—The media of all cultures in a given series, i.e., those cultures of the same medium which had been inoculated on the same day, were combined after harvesting the mycelium. Each L. of medium was subjected to four successive extractions of five minutes duration with 200-ml. portions of chloroform. The four chloroform extracts were combined and allowed to evaporate spontaneously. The small amount of residue was redissolved in 5 ml. of chloroform per L. of medium and tested for presence of disulfiram.

The dried mycelium from each series of cultures was ground to a moderately coarse powder in a mortar, packed into a micropercolator, and extracted exhaustively with chloroform. This percolate was allowed to evaporate spontaneously, and the resulting residue was redissolved in 2 ml. of chloroform per Gm. of mycelium extracted. This concentrated extract was then tested for presence of disulfiram. No disulfiram was detected in either the medium or the mycelium of any of the cultures.

Carpophores of naturally-occurring *C. atramentarius* arising from the same mycelial source as those which had caused typical *Coprinus*-alcohol poisoning in R. Levin were investigated for the presence of disulfiram. A total fresh weight of 6.1 Kg. of mushrooms was processed by a method similar to that reported by Simandl and Franc (6). The mushrooms were allowed to autolyze for two days and were then reduced to a mash in a Waring Blendor. This mash was subjected to four successive extractions of five minutes duration with 300 ml. of carbon tetrachloride per Kg. of fresh mushrooms. The solids were separated from the solvent after each extraction by centrifugation. The combined carbon tetrachloride extracts were filtered and allowed to evaporate spontaneously. The fatty residue remaining was mixed with clean sand, packed into a glass percolator, and extracted exhaustively with anhydrous methanol. The methanol percolate was evaporated to dryness under vacuum at 30°. The slight gummy residue obtained in this way was redissolved in 10 ml. of anhydrous methanol per Kg. of fresh carpophores processed. No crystalline material was obtained. These extracts were purified by chromatographing in the manner previously described and eluting with anhydrous methanol the area which corresponded to the area where known disulfiram appeared on the same chromatograms. These purified extracts were then rechromatographed. No disulfiram was found in any of the carpophore extracts.

[1] Authentic disulfiram supplied through the courtesy of Ayerst Laboratories, New York, N. Y.

In order to ascertain if the extraction procedures which were employed would actually recover disulfiram, a quantity of that compound was added to a quantity of naturally-occurring carpophores of *C. atramentarius*. An 800-Gm. quantity of fresh mushrooms was reduced to a mash in a Waring Blendor, and 0.01% of disulfiram was added to one-half of the mash. After two days both portions of the mash were processed. The portion to which no disulfiram was added gave no test for disulfiram. The portion to which disulfiram was added was extracted, and the residue was dissolved in 10 ml. of anhydrous methanol. Fifty microliters of this concentrated extract were chromatographed and gave a color reaction with saturated cuprous chloride solution of greater intensity than 100 mcg. of known disulfiram. This indicated that the 10 ml. of extract contained over 20 mg. of disulfiram. The extraction procedure was therefore more than 50% efficient.

DISCUSSION

The quantities of mycelium produced by *C. atramentarius* when cultured on the seven media employed were extremely small. Medium No. 7, the horse dung extract medium, gave a slightly higher yield of the fungus than any of the other media tested. The small darkened areas of mycelial compaction which were observed when the fungus was grown on solid medium were similar to the structures described by C. H. Chow (8) as rudimentary carpophores; however, no true carpophores were produced.

The processes of extraction employed were shown to have an efficiency of at least 50%, and the chromatographic technique which was used could readily detect a 100-mcg. quantity of disulfiram. Consequently, the extracts of the culture media must have contained less than 0.2 mg. % of disulfiram or that compound would have been detected. Similarly, the mycelium extracts must have contained less than 80 mg. % of disulfiram, and the extracts of the naturally-occurring carpophores must have contained less than 0.4 mg. % of disulfiram (fresh weight basis). If disulfiram were present in the carpophores at a level just below that which was detectable by the procedures employed, approximately 125 Kg. of fresh mushrooms would have to be consumed in order to obtain the ordinary daily maintenance dose of 500 mg. of disulfiram (20). *C. atramentarius* arising from the same mycelial source as that investigated by the authors is known to possess disulfiram-like physiological activity when eaten in ordinary amounts. As the maximum possible level of disulfiram in the carpophores investigated by the authors is far below that which could exert physiological activity, it must be concluded that the disulfiram-like activity of these carpophores is due to some other agent present in the fungus.

The fact that disulfiram did not exist in the *C. atramentarius* investigated does not entirely rule out the possibility that a recoverable concentration of that compound did occur in the fungus investigated by Simandl and Franc (6). Such variation could be due to several factors. First, the positive identification of the black-spored Agarics is extremely difficult. One cannot be positive that the "*Coprinus atramentarius*" investigated by Simandl and Franc is identical with the organism investigated here. However, such a discrepancy is rather unlikely. Second, nutritional and environmental factors can alter the metabolism of organisms in such a way as to change, at least quantitatively, the products of that metabolism. Third, various genetic strains of *C. atramentarius* may exist which differ appreciably in their metabolism. The features upon which the taxonomy of the Coprini is based are chiefly morphological and anatomical in nature. Very little is known of the genetics of these organisms, and more than one genotype may well exist within a single phenotype.

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